Hydrogenotrophic Methanogenesis under Alkaline Conditions.

Running Title - Hydrogenotrophic methanogenesis dominates above pH 9.0

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Abstract

A cement based geological disposal facility (GDF) is one potential option for the disposal of intermediate level radioactive wastes. The presence of both organic and metallic materials within a GDF provides the opportunity for both acetoclastic and hydrogenotrophic methanogenesis. However, for these processes to proceed they need to adapt to the alkaline environment generated by the cementitious materials employed in backfilling and construction. Within the present study a range of alkaline and neutral pH sediments were investigated to determine the upper pH limit and the preferred route of methane generation. In all cases the acetoclastic route did not proceed above pH 9.0 and the hydrogenotrophic route dominated methane generation under alkaline conditions. In some alkaline sediments acetate metabolism was coupled to hydrogenotrophic methanogenesis via syntrophic acetate oxidation, which was confirmed through inhibition studies employing fluoromethane. The absence of acetoclastic methanogenesis at alkaline pH values (>pH 9.0) is attributed to the dominance of the acetate anion over the uncharged, undissociated acid. Under these conditions acetoclastic methanogens require an active transport system to access their substrate. The data indicates that hydrogenotrophic methanogenesis is the dominant methanogenic pathway under alkaline conditions (>pH 9.0).

Introduction

Globally, geological disposal is largely the preferred choice for the management of intermediate level radioactive wastes (ILW) (IAEA, 2011) with a number of countries pursuing the establishment of Geological Disposal Facilities (GDF) (Faybishenko et al., 2016). The exact design of these facilities is dependent on the host geology, however there are often a number of common features including: the use of cementitious construction and backfill materials; the presence of metal in the construction, container and waste components; and organic, primarily cellulose, containing wastes.

The presence of metallic and organic materials means that abiotic and biotic gas generating processes (NEA, 2014) are likely to proceed within a GDF resulting in gas generation being evaluated in a number of international waste management programmes (Felicione et al., 2003; NDA, 2010; Avis et al., 2011; Summerling, 2013; Poller et al., 2016). The anaerobic corrosion of metals will generate molecular hydrogen (NDA, 2010; Libert et al., 2011) which can in turn act as an electron donor for hydrogenotrophic methanogenesis (Libert et al., 2011; Bagnoud et al., 2016). The alkaline conditions generated by the cementitious materials will promote the alkaline hydrolysis of cellulosic wastes (Humphreys et al., 2010). This abiotic process generates a range of cellulose degradation products (CDP) (Rout et al., 2015b; Charles et al., 2019) collectively which are dominated (>70%) by isosaccharinic acids (ISA) (Almond et al., 2012).

Recent research has focussed on the use of lime kiln waste sites to provide an insight into the biodegradation of CDP components under alkaline conditions (Milodowski et al., 2013; Charles et al., 2019). Despite the harsh geochemical environment (pH >10.0); these sites support extensive and diverse bacterial and archaeal populations (Burke et al., 2012; Kyeremeh et al., 2016) capable of a wide range of metabolic and energy generating processes (Bassil et al., 2014; Rout et al., 2014; Rout et al., 2015a; Rout et al., 2015b), including methanogenesis (Rout et al., 2015b). The information that is available for these sites suggests that the hydrogenotrophs dominate the methanogenic populations despite the availability of acetic acid and the absence of competition by other acetate consuming pathways (Charles et al., 2015; Rout et al., 2015b; Kyeremeh et al., 2016).

Given the importance of methanogenesis to gas generation within a GDF, a range of anthropogenic alkaline environments of various ages were investigated to determine the impact of environmental pH on the nature and extent of methanogenic activity.

Materials and methods

Sampling site investigations

The investigation involved sediments from three lime kiln waste sites (≈25 to ≈150 years old) (New Lime sites, designated B, H, T) (Charles et al., 2019), five field kiln sites (Johnson, 2008) (200 to 300 years old) (Old Lime sites, designated LK1, LK2, LK3, LK4, LK5) and four steel industry waste sites (5 to ≈30 years old) (Mayes et al., 2008) (Steel sites, designated CW, CS, RC, SC) (See Supporting Information). In addition, a neutral pH, freshwater anaerobic sediment was employed as a control (Control). In the case of the New Lime, Steel and Control sites, hand cored samples (dia. 30 mm) were taken at depth (~1 m). In the case of the Old Lime sites, samples were taken from a shallow depth of 10-30 cm using a stainless steel trowel. All sampling equipment was surface sterilise using a commercial hypochlorous acid spray (Redditch Medical Ltd, Stockton on Tees, UK). Sterile plastic sample containers were filled to maximum capacity with sediments and where possible with the associated pore waters in order to minimise the presence of oxygen. The pH values of the associated pore waters were determined in situ with a hand-held pH probe (Mettler Toledo, Columbus, Ohio, USA) and the pH of the soil/sediment determined using standard methods (B.S.I 2005).

Microcosm investigations

All microcosms employed in the study were incubated in the dark at 25 °C, the incubation time was dependent on the time taken for the microcosm to generate detectable methane and ranged from 2 to 8 weeks. Initial microcosms employing New Lime and Control sediments were established by mixing a soil/sediment sample (5 g) from each location with 5 mL of sterile, nitrogen purged mineral media (MM) (Table S1 & S2) (B.S.I, 2005) adjusted to pH 10.0 with 4 M NaOH for New Lime microcosms and pH 7.0 for the Control microcosms. Slurries were then transferred to 100 ml crimp top bottles containing 100 mL of MM supplemented with 10 % v/v CDP and a hydrogen:nitrogen (10:90) headspace (BOC Ltd, Guildford, UK). A CDP/hydrogen enrichment was chosen since this had previously been shown (Rout et al., 2014;

Rout et al., 2015a; Kyeremeh et al., 2016) to support a diverse range of microbial processes and provided a source of fermentable substrates, acetate and hydrogen. CDP was prepared as previously described (Rout et al., 2014; Rout et al., 2015a) All microcosm experiments were carried out in duplicate.

On the detection of methane initial enrichments were sub-cultured twice at the starting pH by inoculating sterile microcosms with 5 mL of the previous microcosm suspension (Figure S1). This was carried out to reduce the impact of residual fermentable substrates, mineral components and other electron acceptors (e.g. Iron (III), sulphate) present within the inoculating sediment.

Once methane generation was detected at the starting pH, each sediment was sequentially exposed to a range of pH values ranging from pH 7.0 to pH 12.0 (Figure S1) as described below. Every microcosm that was methane positive was subcultured at the next pH in the series and a hydrogen and an acetate enrichment created at the original pH (Figure S1). The last pH in the series to fail to generate methane was still subcultured into hydrogen and acetate enrichments. These enrichments were performed by transferring 5 mL of the microcosm suspension to a 100 ml crimp top bottle containing 100 mL of MM with either a hydrogen: carbon dioxide: nitrogen (10:10:80) (BOC Ltd, Guildford, UK) headspace or MM supplemented with Na-acetate (30mM) with a 100% nitrogen headspace. A set of blank microcosms were also established as described above with MM, soil and a 100% nitrogen atmosphere.

Chemical analysis

Headspace hydrogen and methane was determined via an Agilent GC6850 equipped with HP-PLOT/Q column with particle traps ((35 m x 0.32 mm x 20 μm), Agilent Technologies, Santa Clara, California, USA) employing thermal conductivity detection. Liquid microcosm samples (1 mL) were also removed over the course of incubation using degassed syringes and needles.

Acetic acid concentrations were determined using Gas Chromatography (Hewlett Packard Ltd, London, UK) with a Flame Ionisation Detector (GC-FID) as described previously (Rout et al., 2015a). To determine the presence of acetoclastic methanogenesis the metabolic inhibitor methylflouride was employed at an initial concentration of 1 % as described previously (Daebeler et al., 2013). Duplicate reactors without inhibitor addition were also prepared and served as a control. Methane produced from CO_2 (m_{CO2}) was calculated from inhibited microcosms and acetate-derived methane was calculated from methane production in uninhibited microcosms ($m_{acetate} = m_{total} - m_{CO2}$).

16S rRNA gene sequencing

Total genomic DNA was extracted from both soil/sediment samples and methanogenic enrichment cultures using the PowerSoil DNA Isolation Kit (Qiagen, Manchester, UK). The enrichment cultures were sampled after 2 weeks of incubation by the centrifugation (10,000 x g) of the microcosm fluid to pellet the cells prior to DNA extraction. The V4 region of the 16S rRNA gene was then amplified by PCR using primers 341f and 805r (Takahashi et al., 2014). Next generation sequencing of PCR products was carried out by ChunLab (South Korea) using the Illumina MiSeq platform. Sequences were then paired and clustered into OTUs using DADA2 employing R3.2.5 (Callahan et al., 2016), before being identified using the MegaBLAST search strategies (Altschul et al., 1997).

Results

CDP fed New Lime microcosms

Within microcosms utilising CDP as a carbon source, the Control enrichments demonstrated a linear reduction in methane production between pH 7.0 and pH 11.0 (Figure 1), whilst the New Lime enrichments demonstrated an optimum pH of pH 9.0 for methane generation and a linear reduction down to pH 12.0. This optimum is below the in-situ pH of the New Lime sites (pH

11.5 to pH 13.0, Table S3), suggesting that the in-situ populations have alternative strategies to manage these harsh pH values (Charles et al., 2017).

Mean methane production values for those microcosms producing methane are presented (Figure 1) in order emphasise the overall trends observed. Across the pH range investigated the microbial populations enriched from the three New Lime sites demonstrated different responses to changing pH with the oldest site (site B (See Supplementary material)) generating methane across the whole range and the other two sites generating methane across narrower pH ranges (Figure S2).

In the Control sediment enrichments the archaeal community composition was composed of almost equal proportions of acetoclastic to hydrogenotrophic methanogens (46%:54% Figure 2) at pH 7.0 and pH 8.0. As the enrichment pH became more alkaline (pH ≥9.0), the community composition shifted towards a more hydrogenotrophic population Figure 2), even though acetate was present in these cultures. The New Lime sediment enrichments were dominated by hydrogenotrophic methanogens (Figure 2) suggesting that the long term exposure to alkaline pH had selected against acetoclastic methanogens in these sediments.

Figure 1

Figure 2

H_2:CO_2 and acetate fed New Lime microcosms

The trend towards hydrogenotrophic methanogenesis at alkaline pH was confirmed by the establishement of hydrogen and acetate enrichments from the CDP fed Control and New Lime enrichments at each pH. Within the H_2:CO_2 Control sediment enrichments, hydrogen consumption was at its highest at pH 7.0 at 243.3 μmol day^{-1}, and became undetectable as pH increased to pH 11.0 (Figure 3A). The associated methanogenic communities were most diverse at pH 7.0 with twelve different methanogenic genera detected (Figure 3B).

176 *Methanobacterium spp.* were the most dominant at this pH, comprising 60.7% of the
177 community; with *Methanoregula spp.* (14.7%) and *Methanosphaerula spp.* (10.2%) also being
178 prevalent within the community. As pH was increased through to pH 10.0, the loss of a number
179 of the genera observed at pH 7.0 coincided with the further dominance of *Methanobacterium*
180 *spp.,* rising to 97.1% of the community at pH 10.0.

181 These findings were reinforced by the H_2:CO_2 New Lime enrichments in which
182 *Methanobacterium spp.* were the dominant taxa detected across all pH (7.0 to 11.0)
183 enrichments (Figure 3B). A negative relationship between hydrogen consumption and
184 increasing pH was seen in these enrichments as pH decreased for pH 9.0 to 11.0, however
185 hydrogen consumption was still detected at pH 11.0 (77.0 µmol day^{-1}). The optimum pH for
186 hydrogenotrophic methanogenesis was pH 9.0 with a hydrogen consumption of 167.5 µmol
187 day^{-1}. The alkaliphilic nature of the methanogenic population in the New Lime sediments was
188 indicated by the fact that the rates of hydrogen metabolism decreased as the pH fell below pH
189 9.0 (Figure 3A). The data here reiterate that the optimum pH for hydrogenotrophic
190 methanogenesis is pH 9.0 for communities of this type and that it is not the availability of
191 substrate from fermentation (such as CDP) that is a limiting factor. As was the case with the
192 CDP reactors, not all the New Lime enrichments were active across the whole pH range (Figure
193 S3).

194 Acetate fed control enrichments contained both acetoclastic and hydrogenotrophic
195 methanogens (Figure 4B). Hydrogenotrophic methanogens are able to generate methane from
196 acetate via Syntrophic Acetate Oxidation (SAO). SAO is an endergonic reaction with a Gibbs
197 free energy of +104.6 kJ mol^{-1} and is therefore energetically less favourable than acetoclastic
198 methanogenesis, however, when the oxidation of acetate is coupled to hydrogenotrophic
199 methanogenesis the total combined reaction is exergonic ($\Delta G0' = -31$ kJ mol^{-1}) (Hattori, 2008).
200 Few SAO bacteria have been described but SAO is associated with members of the phylum

Firmicutes (Hattori et al., 2000; Balk et al., 2002; Westerholm et al., 2010; Manzoor et al., 2018). In the acetate fed Control enrichments (Figure 4A) a linear reduction ($R^2 = 0.97$) in the rate of acetate removal was observed between pH 7.0 and pH 9.0 from 182.7 μmoles day^{-1} to 33.7 μmoles day^{-1}; with a cessation of activity at pH 10.0. *Methanosarcina spp.* contributed 65.1 % of the pH 7.0 and 79.3% of the pH 8.0 Control enrichment populations (Figure 4B). This fell to 7.1% within the pH 9.0 enrichment. The fall in acetate consumption between pH 7.0 and 8.0 coincided with the loss of both *Methanospirillium* and *Methanosaeta spp.* and a reduced proportion of *Methanosarcina* between pH 8.0 and 9.0. The loss of *Methanospirillium spp.* may suggest that SAO was active at pH 7.0 in these systems but was not able to adjust to the increase in pH.

Low levels of acetate consumption were only observed at both pH 8.0 (36.4 μmoles day^{-1}) and pH 7.0 (23.6 μmoles day^{-1}) in acetate enrichments from two of the three New Lime sites. However, the methanogenic populations in these enrichments were dominated by the hydrogenotrophic (Liu and Whitman, 2008; Dridi et al., 2012) *Methanobacterium, Methanoculleus* and *Methanomassiliicossus spp.,* with the potentially acetoclastic *Methanosarcina spp.* representing only 1.2% of the total archaeal populations at these pH values (Figure 4B). However, members of the phylum Firmicutes which comprised 30 to 40% of the total populations of the pH 8.0 and 7.0 New Lime acetate enrichments suggesting that SAO may be responsible for the low levels of acetate removal in these enrichments.

Figure 3

Figure 4

Inhibitor studies

To investigate the role of in the acetate fed enrichments, the pH 7.0 acetate fed New Lime enrichments were sub-cultured in the presence of acetate and fluoromethane, an inhibitor of

acetoclastic methanogenesis (Agneessens et al., 2018). Within these enrichments (Figure S4); acetate removal was observed in both the presence and absence of inhibitor (removal rates: Presence: 4.1 x 10^{-2} ± 4 x 10^{-3} mmol day^{-1}; Absence: 3.5 x 10^{-2} ± 4 x 10^{-3} mmol day^{-1}), suggesting that acetate removal in these sediments was SAO linked. The addition of fluoromethane to pH 7.0 acetate fed Control enrichment saw a cessation of acetate removal, indicating that in this case acetoclastic methanogens was the dominant acetate removal process even though the presence of hydrogenotrophs such as *Methanospirillium spp.* may suggest some SAO activity (Figure S5). These results suggest that the role of SAO in methanogen generation in alkaline environments warrants further study.

Steel and Old Lime microcosms at pH 7 and pH 10

In order to determine the extent to which alkaline pH impacts upon acetoclastic methanogenesis across a wider selection of anthropogenic alkaline environments with more diverse methanogenic populations, sediments from the Steel and Old Lime sites were used to establish microcosms at pH 7.0 and pH 10.0. The Old Lime sediments (n=5) had in-situ pH values closer to circum-neutral pH values suggesting the bulk environment had recovered from exposure to alkaline contamination. However, lime fragments are likely to be present within these sediments suggesting that alkaline microsites will persist. The enrichments from these sediments were similar to the Control sediments, with both acetate and hydrogen consumption being negatively impacted by an increase in pH from pH 7.0 to pH 10.0 (Figure 5). In particular, no acetate consumption or methane production was observed at pH 10.0. At pH 7.0 acetate consumption rates were 2.0 x 10^{-2} (± 7.7 x 10^{-3}) day^{-1} and this coincided with the detection of *Methanoculleus sp.* (70.5%), *Methanosarcina sp.* (16.6%) and *Methanomasiliicoccus sp.* (11.6%). Hydrogen consumption could still be detected within the pH 10.0 enrichments; however, the rate fell from that observed at pH 7.0 (43.1 (± 6.4) µmol day^{-1}) to 17.4 (± 4.6)

249 µmol day^{-1}. At both pH 7.0 and 10.0 *Methanoculleus sp.* dominated the community
250 composition, representing 61.6% and 89.8% of the communities respectively.

251 The pH of the Steel sediments ranged from pH 9.5 to pH 12.9. The enrichment of these
252 sediments with either acetate or H_2:CO_2 demonstrated similar behaviour to that observed with
253 the New Lime sediments, again no acetate consumption was detected at pH 10.0 but was
254 observed at pH 7.0 with a rate of 36.5 (± 2.0) µmol day^{-1}. At pH 7.0 acetoclastic communities
255 from the Steel sites were dominated by *Methanosarcina sp.* (78.8%) with *Methanobacterium*
256 *sp*. comprising 20.6% of the community. In contrast to the New Lime enrichments (Figure 3A)
257 the Steel site sediments (Figure 5) demonstrated a greater rate of hydrogen consumption at pH
258 7.0 (90.8 ± 1.9 µmol day^{-1}) than at pH 10.0 (85.1 ± 3.2 µmol day^{-1}), although these were not
259 significantly different (p = 0.105). The hydrogenotrophic methanogenic microcosms were
260 again dominated by *Methanobacterium sp.*

261 Figure 5

262 Overall the results indicate that hydrogenotrophic methanogenesis is favoured over acetoclastic
263 methanogenesis at alkaline pH (> pH 9.0). This observation is consistent across a wide range
264 of calcium based anthropogenic sites of different origins and ages. Under the prevailing
265 geochemical conditions created at these pH values the acetoclastic methanogenic pathway is
266 unable to access its substrate due to the absence of undissociated acetic acid at these pH values.

267 **Discussion**

268 The data presented here suggests that an alkaline pH >9.0 results in a methanogen community
269 that is dominated by hydrogenotrophs, with populations enhanced by the activity of SAO
270 bacteria. The methanogen populations from high pH and neutral sediments exposed to alkaline
271 pH were both dominated by *Methanobacterium sp.*, and in the case of the New Lime sediment
272 communities, were capable of hydrogenotrophic methanogenesis at pH 11.0. Members of the
273 Methanobacteriales have also been detected within a range of hyperalkaline environments

resulting from serpentinisation processes (Brazelton et al., 2017; Crespo-Medina et al., 2017). A number of alkaliphilic *Methanobacterium sp.* have been isolated to date (Boone et al., 1986; Mathrani et al., 1988; Kotelnikova et al., 1998), the observations made here suggest that this genus is adaptable to aggressive pH changes as observed with Control sediment enrichments, but is also capable of surviving high pH after long term exposure as residents of the initial New Lime sediments. This is in contrast to the Methanomicrobiales such as *Methanocalculus sp.* which are more prevalent in the hypersaline-hyperalkaline Soda lakes (Surakasi et al., 2007; Antony et al., 2013; Sorokin et al., 2015), due to the low salt tolerance of *Methanobacterium sp*. (Boone et al., 1986). In this study, *Methanosarcina sp.* were most impacted in the control sediments exposed to high pH in acetate enrichments. Pure culture studies of Lonar lake *Methanosarcina* indicated a maximum pH of 9.5 for growth upon acetate (Thakker and Ranade, 2002) which suggests that this genus may require a saline rather than a calcium dominated environment to sustain methane generation above pH 9.0.

These observations indicate that the generation of methane through the acetoclastic pathway, although energetically favourable with respect to bicarbonate (Jin and Kirk, 2018), does not proceed under alkaline conditions (>pH 9.0). High pH favours the dissociation of acetic acid to its anion ($CH3COO^-$) preventing transmembrane diffusion (Kröninger et al., 2014). Under alkaline conditions acetate transport into the acetoclastic methanogen cell is therefore reliant on an acetate transporter, which is likely to be less energetically favourable than hydrogenotrophic methanogenesis (Kröninger et al., 2014). Much of the current understanding of methanogen ecology under alkaline conditions has been focussed upon the microbiology of hypersaline, hyperalkaline Soda lakes where methanogenesis is most commonly associated with the metabolism of C-1 compounds being released upon the biodegradation of Cyanobacterial mats (Jones et al., 1998; Sorokin et al., 2017). The current study indicates that methanogenesis in non-saline, calcium dominated alkaline environments should be considered

as a different ecological niche. Within these environments hydrogenotrophic methanogenesis is the most prevalent methanogenic pathway above pH 9.0. This observation is supported by investigations of sediments from thirteen individual sites ranging in age, origin and chemical composition. These observations suggest that under the alkaline conditions generated within a cement based GDF it is the hydrogenotrophic methane generation pathway that will be active rather than acetoclastic methanogenesis.

Conflicts of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. RWM Ltd had no influence on the design or execution of this study or the interpretation and reporting of the data reported in this paper.

Author Contributions

PNH, SR, WM and HG contributed to conception and design of the study. RW carried out the experimental work and PNH, WM, HG, RW and SR participated in the field work. SR, PNH and RW wrote the first draft and all authors contributed to manuscript revision, read, and approved the submitted version.

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Figure 1. Methane production rates in CDP-fed microcosms employing Control and New Lime sediments operating between pH 7.0-12.0. Control microcosms (n=2 at all pH values) had a pH optimum of 7.0 (n=3), in contrast the New Lime microcosms (n=2 pH 7.0, n=4 pH 8.0, n=6 pH 9.0, n=6 pH 10.0, n=2 pH 11.0) were optimal at pH 9.0. The upper pH limit for methanogenesis was pH 10.0 for the Control sediment, this increased to pH 11.0 in the case of the New Lime sediments. No methane generation was detected at a pH >11.0. ('n' denotes the number of replicates analysed).

Figure 2: Methanogen communities in neutral and alkaline soil enrichments. When the neutral pH Control sediment was enriched with CDP the acetoclastic and hydrogenotrophic communities were present in roughly equal proportions at pH 7.0 and 8.0. As the pH increased to pH 10.0, the acetoclastic compostion fell as pH increased, with no methanogen community detectable at pH 11.0. In contrast the alkaline New Lime enrichments were dominated by hydrogenotrophic methanogens irrespective of enrichment pH. (ND- none determined).

Figure 3: Hydrogen consumption rates (A) and methanogen populations (B) in H_2/CO_2 enriched microcosms from Control (n=2) and New Lime (n = 6) soils. An increasing pH negatively impacted upon hydrogen consumption in Control soil enrichments (n=2 at all pH values), whilst New Lime sediments (n=4 pH 7.0, n=4 pH 8.0, n=6 pH 9.0, n=6 pH 10.0, n=4 pH 11.0) demonstrated an optimum pH of 9.0. Across all enrichments, *Methanobacterium sp.* were the dominant genus. ND- none detected. ('n' denotes the number of replicates analysed).

Figure 4: Acetate consumption rates (A) and methanogen populations (B) in sodium acetate enriched microcosms from Control and New lime soils. Increasing pH negatively impacted upon acetate consumption with no acetate consumption observed above pH 9.0 in either the Control (n=2 at all pH values) enrichments or the New Lime enrichments (n=2 pH 7.0, n=2 pH 8.0). *Methanosarcina spp.* and *Methanobacterium spp.* were the dominant genera

494 in control enrichments whilst *Methanobacterium sp.* were dominant in the New Lime
495 enrichments. ND- none detected. ('n' denotes the number of replicates analysed).

496 **Figure 5: Acetate and hydrogen consumption from microcosms prepared using Old Lime**
497 **and Steel site sediments as an enrichment inoculum.** The pattern observed in the Old Lime
498 (n=10) and Steel (n=8) sediments mirrored that seen with the New Lime and Control sediments
499 with acetoclastic methanogenesis absent at alkaline pH. ('n' denotes the number of replicates
500 analysed).